Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens

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Abstract

For decades low doses of antibiotics have been widely used in animal production to promote growth. However, there is a trend to reduce this use of antibiotics in feedstuffs and legislation is now in place in Europe to prohibit their use in this way. As a consequence economically important diseases, such as necrotic enteritis (NE) of chickens, caused by *Clostridium perfringens*, have become more prevalent. Recent research is creating a paradigm shift in our understanding of the pathogenesis of NE and is now providing information that will be required to monitor and control the incidence of NE in poultry.

Removing antimicrobial growth promoters and the rise of necrotic enteritis

For decades antibiotics have been extensively used in animal production worldwide[1] (see text box 1). Added in low doses to the feed of farm animals, they have been shown to increase daily weight gain and conversion of feed into body mass, leading to economic advantages for farmers[2,3,4,5]. However, there is an increasing trend to reduce this use of antibiotics in feedstuffs. There are concerns that the use of antibiotics in the feed contributes to the spread of antibiotic resistance genes by promoting the selection of antibiotic resistant bacteria in animals. Also, waste materials from animals may contain antibiotic residues, resulting in their wider dissemination in the environment. Since January 1st 2006, legislation has been in place in Europe to prohibit the use of antibiotics as growth promoters and in other continents the use of antimicrobial growth promoters in feedstuffs is under debate[6,7,8].

The reduction in use of antibiotic growth promoters has had both expected and unexpected effects. The absence of antibiotic growth promoters is known to result in reduced feed conversion efficiencies[9]; this has had economic consequences for livestock producers but is a manageable problem. More worryingly, some animal diseases have become more widespread – there are emerging diseases arising as a consequence of changes in farming practices. One of the most significant emerging diseases is necrotic enteritis (NE) of broiler chickens, which is caused by *Clostridium perfringens*. The full impact of the reduction in antibiotic growth promoters on the incidence of NE is not yet known because many countries are still implementing policies to reduce antibiotic usage in feedstuffs. However, insight into the extent of the problem can be gained from analyzing data from countries that had

abolished antibiotic growth promoters prior to the EU legislation. In Scandinavia the banning of antibiotic growth promoters was accompanied by a dramatic increase in the incidence of NE in poultry. For example, in Norway, most of the feed companies abolished antibacterial growth promoters in 1995 and within one month increases in the incidence of NE in flocks were evident, indicating that the AGPs had been an effective prophylactic treatment controlling NE. The problem was so great that alternative prescription antibiotics were introduced, although the total quantities of antibiotics used to treat NE were less than those used in feed[10,11]. AGPs continue to be used in many of the major poultry producing regions, including North and South America and Asia. It has been estimated that NE costs the international poultry industry about 2 billion US dollars annually[12,13]. The latest research into the biology of NE is precipitating a reassessment of our understanding of the disease process and is offering new avenues for the development of vaccines to address this important economic issue.

Necrotic enteritis in broiler chickens

C. perfringens produces several myonecrotic and gastrointestinal diseases in humans, domesticated livestock and in birds[14,15]. As a species *C. perfringens* is capable of producing a wide range of toxins (see Text box 2), but individual strains produce only a sub-set of these toxins. The differential production of the four so-called major toxins (α-, β-, ε- and ι-toxin) is used to classify strains into five toxinotypes (A, B, C, D and E)[16]. In poultry, both clinical and subclinical NE are typically caused by *C. perfringens* type A[17]. Of the major typing toxins, type A strains produce only α-toxin, which is responsible for the ability of *C. perfringens* strains to cause gas gangrene[18].

Since spores of *C. perfringens* type A are ubiquitous in the environment and are ingested continuously via poultry feed, it is generally accepted that predisposing factors are required for these bacteria to cause disease. Complex interactions between members of the gut flora play a role in establishing NE. The best known predisposing factor is pre-existing mucosal damage caused by coccidiosis[19,20,21]. Coccidiosis is caused by *Eimeria* parasites that colonize the gut and kill epithelial cells as a consequence of the intracellular stages of their life cycle. The resulting gaps in the epithelial lining of the intestinal lumen may trigger rapid replication and/or toxin production by certain *C. perfringens* strains.

Necrotic enteritis is characterised clinically by a sudden increase in flock mortality, often without premonitory signs although wet litter is sometimes an early indicator of disease. At necropsy large necrotic foci are found in the small intestinal mucosa (Fig 1a) and in severe cases the whole mucosal surface of the gut is affected, with extensive necrosis of the lumen surface (Fig 1b). The typical form of the disease leads to increased mortality in broiler flocks during the last weeks of the rearing period[20]. In the past few years, for reasons that are not clear, *C. perfringens* has been associated with a form of NE with clinical signs that are milder than the classical acute form of NE[22,23,24]. In this subclinical form, *C. perfringens* causes chronic damage to the intestinal mucosa, which leads to decreased digestion and absorption, reduced weight gain and increased feed conversion ratio[25,26] It is this manifestation of the disease that reportedly causes the greatest economic losses in the poultry production industry[27].

Histopathology of NE lesions

Early microscopic studies of NE lesions described clearly demarcated foci of advanced tissue degeneration with villi partially or fully denuded of epithelium, large sheets of disintegrating cells found in the lumen and congestion of blood vessels in the lamina propria and submucosa[28,29]. Recent studies of earlier stages of disease have provided valuable new insights into the pathogenesis of NE, indicating damage to the villi initially occurs in the basement membrane and lateral domain of the enterocytes, spreading throughout the lamina propria. Epithelial damage occurs later in the process[30]. Olkowski *et al.* (2008)[30] concluded that the morphological changes indicated that the initiation of pathology involves factors affecting the extracellular matrix and cellular junctions. It was suggested that the pathology may be the result of bacterial collagenases, whose action is enhanced when mucosal damage (e.g. induced by coccidia) is present, or host matrix metalloproteinases that are activated by the host-pathogen interaction[30]. It is interesting to note that one of the recently identified, potentially protective, vaccine antigens from *C. perfringens* may be a zinc metallopeptidase[31].

Do *Clostridium perfringens* toxins play a role in NE?

For more than 20 years, α-toxin has been proposed to be the main virulence factor for NE in poultry. The origin of this assumption is unclear, but seems to lie in the observations that crude supernatant from *C. perfringens* type A cultures induced necrotic lesions in broilers[32] and that serum containing antibodies to *C. perfringens* α-toxin prevented the development of lesions[33]. Maternal vaccination with a crude *C. perfringens* type A and C toxoid has been

shown to induce antibodies against α-toxin in chicks, which were partially protected against NE[34]. The interpretation of these early studies is unclear because they used crude supernatant and the assumption was made that the effects were caused by the dominant protein present, i.e. α-toxin. However, many other proteins are also present in the supernatant of *C. perfringens* cultures. More recently, other epidemiological and experimental evidence has supported the proposal that α-toxin is an important protective antigen. Two independent studies have shown that poultry that are immune to NE have high titres of antibodies to α-toxin[35,36]. Recently, more convincing evidence of the possible involvement of α-toxin has come from immunization with purified α-toxoid, which induced protection against experimentally induced NE[31].

Against this background, other recent studies have clearly indicated that α-toxin plays no direct role in the pathogenesis of NE. *C. perfringens* isolates associated with NE and isolates derived from the microbiota of normal broilers are predominantly type A[37]. There is no apparent difference in the levels of α-toxin production *in vitro* by normal microbiota and outbreak isolates[38]. In fowl models of NE the disease can be reproduced by type A strains from outbreaks of NE but not by other type A strains, even though both groups of isolates produce α-toxin[39]. However, the most convincing evidence of the lack of involvement of α-toxin in disease comes from studies using an α-toxin negative mutant of a *C. perfringens* strain from an NE outbreak. In virulence trials both the wild-type strain and an isogenic α-toxin negative mutant were able to cause NE lesions in chickens[39]. In contrast, in another study spontaneously derived α-toxin mutants of a virulent strain clearly had an impaired ability to cause NE lesions[40]. However, these mutants were not complemented with a functional α-toxin

gene so it was not clear that the reduced virulence was due to the impairment of α-toxin production.

Other observations also argue strongly against a role for α-toxin in NE. The massive inflammation caused by granulocytes transmigrating from the tissue into the lumen is a hallmark of broiler NE[38,41]. It is markedly different to the leukostasis and lack of inflammation induced by α-toxin in gas gangrene[42]. Indeed, α-toxin negative mutants of *C. perfringens* do promote profound inflammatory responses and are unable to cause gas gangrene in mice[18]. Thus the massive immune cell influx in NE lesions seems to be inconsistent with the known effects of α-toxin on the innate immune system[38,41]. Further, histological analysis of tissue damage occurring in early stages of lesion development is not consistent with the phospholipase C or sphingomyelinase activities of α-toxin[30].

With evidence mounting that α-toxin did not play a major role in the pathogenesis of NE there was a need to reevaluate previous work and an opportunity to search for other factors important in pathogenesis. The earlier evidence used to suggest a role for α-toxin is still valuable because it did indicate that there are molecules in *C. perfringens* culture supernatant that when infused into gut reproduced disease-like pathology[32]. This result showed that significant virulence factors must be present in the secreted cell products.

NetB, the new toxin on the block

Recently, NetB, a novel toxin that is associated with broiler NE has been described[43]. The toxin was identified using screens for proteins from the supernatant of *C. perfringens* cultures

that were cytotoxic for chicken hepatocellular carcinoma cells (LMH) *in vitro*. The *netB* structural gene was identified by genomic sequencing of an NE isolate[43]. Both native and recombinant NetB were shown to be cytotoxic for LMH cells and the mechanism of action appears to involve the formation of small hydrophilic 1.6 -1.8 nm pores[43]. A *netB* mutant of *C. perfringens* was unable to cause necrotic lesions in the gut of experimentally infected broilers, but a complemented *netB* mutant was virulent, like the wild-type strain[43]. Additional evidence for the role of NetB in disease comes from the finding that most NE outbreak strains carry the *netB* gene, whilst *C. perfringens* isolates from other diseases of animals lack this gene and therefore do not produce NetB toxin. This finding has been confirmed and extended by the authors of this review for isolates from Denmark and Belgium, where eight out of eleven isolates from diseased chickens carried the *netB* gene, compared to one out of thirty-two isolates from healthy broilers. However, this story may yet have some further twists to the tail. It is unclear whether the occasional NE isolates that lack the *netB* gene posses a sequence divergent from that of the published toxin or whether other as yet unidentified toxins can confer disease-causing potential on *C. perfringens* isolates. A further complication is that not all strains isolated from diseased birds are necessarily still pathogenic, for instance Thompson *et al.* (2006) [40] found that two of the six isolates they tested were not pathogenic. Hence, caution is required when interpreting surveys of isolates from disease outbreaks; isolates may change during the culturing process or there may even be a mixture of pathogenic and non-pathogenic stains present in some diseased birds.

It is possible that other virulence factors, such as hydrolytic enzymes and other (unidentified) toxins also play a role in the complex pathogenesis of the disease. For example, it has been

suggested that in the initial stages of NE proteolytic enzymes play an important role, causing disruption of the basal lamina matrix and the lateral domain of enterocytes[30]. Indeed, in broilers undergoing NE, the extracellular matrix is disorganized and can even be completely absent.

Vaccination with α-toxin

Until recently, most vaccination efforts directed against NE have concentrated on the use of culture supernatant toxoids in which α-toxin was the major component[34,44,45]. These trials have been of limited success. More recently, vaccines using recombinant α-toxin and live delivery of α-toxin by attenuated *Salmonella* have been evaluated and shown to give partial protection against NE in experimental challenge models[31,46,47]. With evidence accumulating that α-toxin may not play an important role in NE pathogenesis the question arises as to how antibodies to the protein can offer any level of protection. α-toxin is the major protein found in *C. perfringens* culture supernatants and so it was always regarded solely as a secreted protein. As such it was difficult to see how antibodies to the protein could affect disease outcomes if α-toxin was not an important factor in pathogenesis.

Insight into the role in protection of antibodies to α-toxin comes from a recent study by Zekerias *et al.* (2008)[47]. These researchers found that serum from broilers that had been vaccinated with a *Salmonella enterica* subspecies Typhimurium Δ*asd pabA pabB* mutant secreting the C-terminal (non-toxic) domain of α-toxin bound to the cell surface of *C. perfringens* and suppressed its growth in broth cultures. This result clearly shows that although α-toxin is primarily secreted some toxin must also be retained on the cell surface.

Antibody binding may have subsequently blocked some *C. perfringens* cellular process or interaction with the host[47]. There are important parallels here with findings from other bacteria species, where antibodies to ABC transporter proteins, which are not virulence factors, can provide protection against disease, possibly by interfering with the transport of materials into or out of the cell[48]. It is clear that α-toxoid can be used as a protective antigen to vaccinate broilers, but this does not automatically indicate a primary role in the pathogenesis of the disease.

Identification of other vaccine antigens

Thompson *et al.* (2006) [40] have used attenuated strains of *C. perfringens* that no longer express α-toxin to vaccinate chickens. They found that the attenuated vaccine strains offered varying levels of protection. This study clearly demonstrated that *C. perfringens* carries other molecules that can provide some efficacy as vaccine antigens. Subsequent work by this group has identified several immunoreactive proteins in virulent *C. perfringens* strains that, in recombinant subunit form, offer some protection when used as vaccines[31]. Two of these proteins, fructose-biphosphate-aldolase, and a hypothetical protein (possibly a metallopeptidase) also gave some protection when delivered by a live *Salmonella* vector[48]. Other proteins that have been shown to play a role in pathogenesis will be well worth testing as vaccine antigens. These include the NetB protein identified by Keyburn *et al.* (2008)[43] and the metalloproteinases that are suggested to have a key role in early lesion development[30].

Conclusion

The story of NE in intensively reared poultry is fascinating and highlights some of the pitfalls in both policy and in research. The abolition of growth promoters in feedstuffs was initially accompanied by significant increases in disease in animals. In part, this change in the pattern of disease reflects the ways in which farming practices have become interdependent and the difficulties associated with isolated changes in these practices. The story of NE also highlights the dangers of making scientific assumptions and the ease with which such assumptions can become embedded in the literature as fact. However, the story is not one only of pitfalls and problems. The research carried out over the past few years is creating a paradigm shift in our understanding of the pathogenesis of this disease and is now providing the essential information that will be required to monitor and control the incidence of NE in poultry. As new tools, such as relatively cheap whole genome sequencing, targeted mutation and improved disease models are developed we are better able to investigate issues such as the complex etiology of this multifactorial disease. We can look forward to major advances in our understanding of the impact of various genetic and environmental changes on the host-pathogen interaction and how this affects the incidence and severity of disease. Deeper understanding of these issues should facilitate the development of effective disease control measures.

TEXT BOX 1. What are antimicrobial growth promoters ?

Antimicrobial growth promoters are substances that are added to the feed in sub-therapeutic levels in intensive animal rearing (poultry, pigs, cattle) to improve weight gain and conversion of feed into body mass[7,50]. Reduced mortality and morbidity due

to subclinical and clinical disease is also achieved[7,50]. The mechanism of action of antimicrobial growth promoters is unknown, but most likely their action is mediated by their antibacterial effects[7]. It is believed that there is increased nutrient availability to the host when the number of gut bacteria is decreased[51]. Furthermore, the production of toxins by gut bacteria is diminished with consequent beneficial effects on the gut wall integrity[51]. Concerns over the development of bacterial antibiotic resistance and thus the impairment of the efficacy of therapeutic drugs for humans have led to recommendations on the reduction and elimination of the use of antimicrobial growth promoters as animal feed supplements[8,52]. In the EU, the use of antimicrobial growth promoters in animal feed has been banned since 1st January 2006[6]. However, although antibiotics have been withdrawn, coccidiostats are still in use and these agents also have some antimicrobial activity[6]. In the US, there is little regulatory activity regarding the use of these substances, but consumer concerns are influencing debates on the use of antimicrobial growth promoters.

TEXT BOX 2; *Clostridium perfringens* toxins

***C. perfringens* is able to cause a wide range of diseases in humans and animals, ranging from food poisoning to severe invasive disease[15,53,54]. The ability of *C. perfringens* to cause disease is ascribed mainly to the differential production of four major and 10 minor protein toxins[15,55]. The roles of some of these toxins in disease is well understood. For example, α-toxin, which is produced by all five toxinotypes, is the main virulence determinant in gas gangrene and mutated strains that are unable to produce α-toxin are unable to cause disease in mice[18,54]. The ε- and β-toxins appear to**

play key roles in enterotoxaemia in calves, lambs, piglets and goats and most of the domesticated livestock in developed countries are immunised against disease with toxoid vaccines. The *C. perfringens* enterotoxin, CPE, is a major cause of human food poisoning in the USA and Europe[53,56,57]. The roles of the other toxins are less well understood, indeed some may not play a role in disease. The most recently discovered toxins are the beta-2 toxin and NetB toxin. The beta-2 toxin has not been shown conclusively to play a role in disease, but NetB toxin is now known to play a major role in the pathogenesis of necrotic enteritis in poultry[43,58,59,60].

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Fig. 1. Typical gut lesions in severe broiler necrotic enteritis, (a) consisting of patches of necrosis throughout the gastrointestinal tract and in extreme cases (b) extensive necrosis of the mucosal surface.